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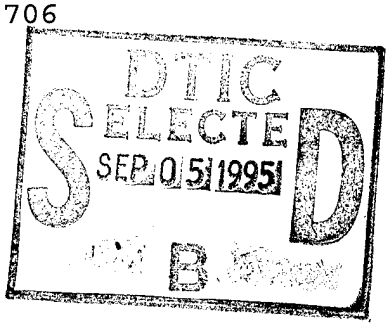

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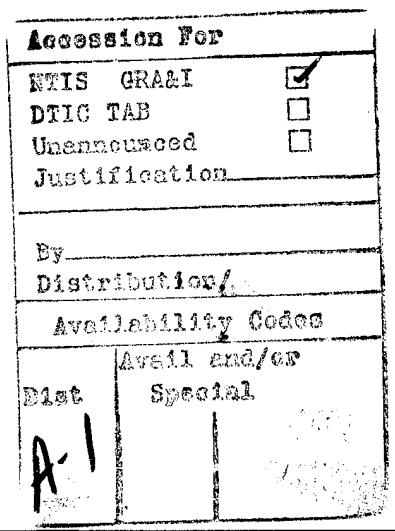

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TABLE OF CONTENTS

ABSTRACT

Inbred strains of rats differ in their susceptibility to both spontaneous and chemically-induced mammary carcinoma formation. Classical genetic breeding studies combined with modern molecular biology techniques can be utilized to identify the gene or genes responsible for these inherited differences. We are currently using such techniques to try to identify the gene(s) responsible for the mammary carcinoma resistance trait in inbred Copenhagen (COP) and Wistar Kyoto (WKy) rats. Previous data identified a marker on rat chromosome 2 which is linked to the resistance phenotype. However further markers must be obtained to map the region adequately before chromosome walking can begin and attempts made to isolate the gene. To this end we are applying several techniques. First, new simple sequence repeat markers are being used to further map the rat genome and look for other regions involved in the resistance phenotype. This approach has yielded a region on rat chromosome 7 which initially appears linked to the resistance phenotype. However closer markers must be identified to confirm this linkage. Two other techniques are being employed to further our progress towards mapping of this phenotype. Microdissected and chromosome-sorted libraries for rat chromosome 2 are being generated and screened for simple sequence repeat markers in an attempt to better characterize the region on chromosome 2. Also, a new technique called genetically driven representational difference analysis (GDRDA) is being employed to isolate new markers throughout the genome which are linked to the phenotype.

INTRODUCTION

It has been known since the 1950's that different strains of laboratory rats exhibit different rates of formation of various kinds of tumors (Dunning and Curtis, 1945). The Wistar Furth (WF) and Fisher 344 (F344) strains are highly susceptible to both spontaneous and chemically-induced mammary tumors (Gould, 1986). The Copenhagen (COP) and Wistar Kyoto (WKy) strains are very resistant to both types of mammary cancer (Dunning and Curtis, 1945; Haag *et al.*, 1992). The resistance to mammary carcinomas in the Copenhagen rat was initially described in the 1940's. Using the chemical carcinogen 2-acetylaminoflourene (AAF), it was shown that Copenhagen rats rarely developed mammary cancers but were not protected from the formation of hepatic cancers. Thus the cancer resistant phenotype of these rats was believed to be mammary specific (Dunning and Curtis, 1945). Research completed within the last ten years by both this laboratory and that of Dr. John Isaacs pinpointed the nature and site of action of the genes involved in this phenotype. Using classical genetic breeding studies and transplantation studies it was demonstrated that the mammary carcinoma resistance phenotype of the COP rat was likely due to the action of a single autosomal dominant gene whose site of action lies within the mammary epithelium (Isaacs, 1986; Gould *et al.*,1989; Haag *et al.*, 1992).

The goal of this research is to map and eventually clone the rat gene(s) we call MCS (mammary carcinoma suppressor) which are responsible for the tumor

resistance phenotype in the hope it will prove useful as a diagnostic indicator of breast cancer risk in humans and possibly lead to the formation of new drugs for the treatment of human breast cancers. To this end much research has already been completed. Using four strains of rats, two which are susceptible to chemically-induced mammary carcinogenesis (WF and F344) and two which are resistant (COP and WKy), two separate backcross sets of animals were generated and tested for the formation of mammary tumors following administration of 7,12-dimethybenz (a) anthracene (DMBA). For each cross, (WKy x F344)F1 x F344 and (COP x WF)F1 x WF, approximately 200 female offspring were generated and phenotyped. Animals containing two or more mammary tumors were genotyped as lacking the tumor suppressor gene, animals having no tumors as carrying the suppressor gene, and animals with one mammary tumor were considered ambiguous (Hsu *et al.*,1994). Using the relatively new techniques of mapping with mini- and microsatellite markers (Jeffreys *et al.*, 1985; Hilbert *et al.*, 1991; Jacob *et al.*, 1991) a linkage map was created for the two backcross sets. Briefly mini- and microsatellite markers are composed of simple sequence repeats (SSR) 1-60 base pairs in length and often contain dinucleotide repeats (Miesfeld *et al.*, 1981; Hamada *et al.*, 1982). These repeats are located throughout the genome at roughly 30 centimorgan distances (Hamada *et al.*, 1982; Stallings *et al.*, 1991), and the length of each repeat can vary from strain to strain thus making them useful for genetic mapping (Weber, 1990). Microsatellite markers are distinguished on sequencing gels following a polymerase chain reaction (PCR) using primers located on either side of the repeat,

while minisatellite markers are resolved on genomic Southern blots. Genetic mapping of the MCS phenotype using these SSR markers and our two sets of backcross animals was performed in two different ways. First, qualitative analysis was performed under the assumption that tumor resistance was caused by a single locus having two alleles, with COP and WKy rats having the genotype SS (dominant suppressor), and WF and F344 having the genotype ss (recessive allele = susceptible). For the backcross analysis, resistant animals (no tumor formation) were assumed to be heterozygous (Ss) for the locus and susceptible animals (two or more tumors) were assumed homozygous (ss). Second, quantitative analysis was performed in order to allow for the possibility that resistance might be due to more than one gene locus. In this analysis the number of tumors is dealt with as a quantitative trait and estimates the contribution of a given locus to the phenotype (Lander *et al.*, 1987). Using both types of analysis, the minisatellite marker M13 was shown to be linked to the MCS phenotype (Hsu *et al.*, 1994). M13 yields a LOD score of 4 using qualitative analysis and 3.8 using quantitative analysis. A LOD score of 3 indicates that the marker has a greater than 99% chance of being linked to the specific trait. Fluorescence *In situ* hybridization (FISH) to whole rat chromosomes using a P1 clone containing the sequence of a marker (*Mit-R1025*), which lies near M13, confirms mapping to the centromeric end of rat chromosome 2 (Hsu *et al.*, 1994). The mapping distance from M13 to the mammary carcinoma suppressor locus (*Mcs-1*) was tentatively identified at 28 cM from R1025 on the centromeric end of chromosome 2 (Appendix A). As this distance is based on the

recombination fraction of only one marker and under the assumption that there is only one suppressor gene, this distance cannot be believed as accurate therefore we have not yet pinpointed *Mcs-1* on the map pinpointed in Appendix A. If other loci were involved the perceived distance would appear larger than the actual distance since the effect of additional loci will be incorrectly attributed to recombination events between the marker and the trait locus.

Initial studies of a related experiment involving mammary tumor formation in (COP xWF)F1 hybrids demonstrated that 27% of the animals developed tumors following DMBA exposure unlike the 0% (as in the parental COP strain) which was expected since each animal contained one copy of the tumor suppressor (Gould *et al.*, 1989). With the (WKy xF344)F1 hybrids, ten percent of the animals developed mammary tumors in comparison to the parental WKy (3%) (Haag *et al.*, 1992). We believe these results are due to loss of the tumor suppressor gene following DMBA exposure. Preliminary data in the primary tumors demonstrates that 12 of 33 microsatellite markers tested displayed loss of heterozygosity (Hsu *et al.*, in preparation). Surprisingly, none of the chromosome 2 markers demonstrated any loss in this experiment, suggesting that chromosome 2 deletions may not be involved in the formation of mammary carcinomas or that the markers used were not sufficiently close to the *Mcs-1* gene to identify such losses. Further mapping of MCS alleles will allow these experiments to be repeated using better markers.

Mcs-1 is believed to represent a new tumor suppressor gene since the tumor

suppressor gene *RB1* maps to rat chromosome 15 (Szpirer *et al.*, 1991) and *p53* (Thompson *et al.*, 1990; Lindblom *et al.*, 1993) and *BRCA1* (Hall *et al.*, 1990) both likely map to rat chromosome 10 based on estimates of known homologous regions between rat and human chromosomes (Levan *et al.*, 1991). Recent mapping experiments in this laboratory confirm the location of *BRCA1* on rat chromosome 10 (unpublished results).

The main objectives of this proposed research were as follows:

1. Isolate new simple sequence repeat markers to fine-map (to within 1 cM resolution) the region of chromosome 2 surrounding the *Mcs-1* locus.

2. Test additional markers over the thus far untested regions of the rat genome for linkage to the MCS phenotype (to 5 cM resolution). Confirm mapping of additional genes to specific chromosomes by FISH to whole chromosomes using specific SSR-positive P1 clones.

3. Fine-map new areas in the genome (to 1 cM resolution) that demonstrate linkage to the MCS phenotype. Begin to positionally clone the gene(s) identified.

4. Isolate coding sequences from P1 or cosmid clones containing MCS phenotype-linked SSRs for determination of homologous regions in the human genome and possible correlation with already identified human genes.

5. Test for loss of heterozygosity of MCS-linked SSR markers in mammary tumors of two independent F1 hybrids following radiation and DMBA tumor induction.

The past year has been spent trying to obtain additional SSR markers from the chromosome 2 libraries, screening the entire rat genome for linkage to the MCS phenotype with new SSR markers obtained from Research Genetics, and utilizing a new technique, Genetically Driven Representational Difference Analysis (GDRDA), to obtain new genomic markers that are linked to the tumor suppressor phenotype. GDRDA is a PCR-based subtraction procedure that leads to the generation of restriction fragment length polymorphisms (RFLPs) markers that are closely linked to the phenotype of interest (Lisitsyn *et al.*, 1993; Lisitsyn *et al.*, 1994). For this procedure two libraries (tester and driver) are constructed from restriction endonuclease digested DNA to which single-stranded adaptors are ligated so that PCR can be used to create a large amount of each library. Under the conditions used for PCR only small fragments (roughly 0.15 -2.0 kb) are generated. Thus each library is only a representation of the original DNA sample (i.e. only a subset of the total restriction endonuclease fragments are present in the library). The DNA samples used for our analysis came from the (COP x WF)F1 x WF backcross animals. For our analysis the tester library was constructed from COP DNA. This sample contained one copy of the *Mcs-1* gene. The driver library was constructed of a mixture of DNA from 20 different backcross animals, all of which exhibited the formation of four or more mammary tumors following DMBA exposure. Importantly,

these animals should lack the *Mcs-1* gene. Twenty animals were used so that the representation of all other alleles in the genome would be approximately 50% COP and 50% WF (Lisitsyn *et al.*, 1994). After each library is constructed the PCR adaptors are removed from both libraries. For the tester library the DNA is size-selected and new adaptors are added to the resulting sample. Then the tester library is mixed with an excess of the driver library, denatured and allowed to anneal. During the annealing reaction three complexes are formed: driver to driver complexes, driver to tester complexes, and tester to tester complexes. At the end of the annealing time PCR is performed using primers for the tester library adaptors. Since only the tester to tester complexes have adaptors on each end, they only will be exponentially amplified by PCR. Three successive rounds of annealing/amplification are performed and the resulting DNA fragments are subcloned into a pUC 19 vector followed by transformation into bacterial cells. Individual clones from this library are then screened by Southern analysis for the proper attributes. A desired clone would hybridize to the original tester library but not the driver library and would exhibit a RFLP between the two parental rat strains. Due to the way in which this experimental system is designed we expect the RFLP pattern to show a small fragment in the COP DNA and a large fragment in the WF DNA. Because the original libraries are generated from the whole rat genome this method of generating probes should help us isolate both new genomic markers linked to *Mcs-1* on chromosome 2 and other markers linked to the phenotype which may be located at other positions in the rat genome.

It should be noted at this point that all of the data reported in this document were not produced solely by Dr. Ford. Dr. Ford spent the last year working out the conditions for the GDRDA approach and utilizing it in an attempt to gain new markers for the resistance phenotype. The generation of chromosome-specific and microdissected libraries was performed by Dr. Laurie Shepel and the SSR mapping was performed by Gerlyn Heil. Both Dr. Shepel and Miss Heil collect salaries from separate sources. While Dr. Ford did not specifically conduct the work of Dr. Shepel and Miss Heil, she was involved on a weekly basis with the progress and design of these experiments. As with most large mapping projects, this one is a team effort.

BODY

MATERIALS AND METHODS

Generation of Chromosome 2 Libraries

For whole chromosome 2 libraries, the source DNA is obtained by flow cytometric sorting of rat chromosome 2 (Shepel et al, 1994), and the chromosomes are used directly for PCR without DNA purification. Microdissected regions 2q1 and 2q1.1 of chromosome 2 were obtained from BIOS Labs, New Haven, CT.

PCR was carried out using the degenerate primer 5'CCGACTCGAGNNNNNNATGTGG 3' (Telenius et al, 1992). The other primer listed in the initial proposal, 5'CCCAAGCTTGCATGCGAATTCNNNNCAGG3', was not used after the initial attempts because it yielded PCR products that were too specific and

thus not a random representation of the template. Template for each reaction consisted of 1000-2000 copies of whole chromosome 2, 10 copies (5 dissections) of each microdissection, and 20 ng of Copenhagen DNA for a positive control. A negative control reaction containing no DNA was also performed to ensure no background contamination. Reaction volumes were 25 μl for the positive and negative controls as well as the microdissected DNAs, and 50 μl for the sorted chromosome template. Final concentrations of reagents were 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs, 1.5 μM primer, 1.25 Units Taq LD polymerase (Perkin-Elmer Cetus). Note also that for whole sorted chromosomes, there will be additional components in the reaction which come from the sheath fluid in which the chromosomes are sorted (some salts and spermine and spermidine), but these do not seem to inhibit the reaction. We followed the protocol of Guan et al. (1992) with the following cycle conditions. The reaction mixtures were first heated to 94°C for 4 min, followed by one cycle at 94°C for 1 min, 20°C annealing for 1 min, and 72°C synthesis for 3 min, then 9 cycles at 94°C for 1 min, 20°C for 1 min, 72°C for 1 min, and 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min with a 5 sec auto-extension on each synthesis step. The last 72°C synthesis step was carried out for 10 min.

To prepare the reaction products for insertion into the pAMP10 vector (Gibco BRL Life Technologies, Gaithersburg, MD), 2.5 μL of the amplified product was reamplified with the same primer bearing a $(CUA)_4$ tail at the 5' end. For a 100 μL reaction volume the mixture was heated to 94°C for 5 min followed by 20 cycles at 94°C for 1 min, 50°C for 1 min, and

72°C for 1 min. The final synthesis step was extended to 10 min. Two μL of this product were annealed into the pAMP10 vector (20 μL reaction volume) according to the manufacturer's directions (CloneAmp Systems, Gibco BRL). One μL of the annealed reaction was used to transform DH5αF' competent cells (Gibco-BRL Life Technologies, Gaithersburg, MD) yielding 2000-4000 transformants grown under ampicillin selection.

Screening and sequencing of Chromosome 2 libraries.

Transformants containing chromosome 2-specific sequences were screened for $(CA)_n$ repeats by colony filter hybridization with a poly (dA-dC)/poly(dG-dC) polymer (Pharmacia, Piscataway, NJ). We have successfully used both radioisotope-labelled as well as biotin-labelled (Rad-Free system, Schleicher and Schuell) probe for this screening. Positive clones were isolated and either plasmid DNA or ssDNA was isolated for sequencing. DNA sequencing to identify the unique DNA sequences surrounding the $(CA)_n$ repeats was performed using the Sequenase system (USB). New primers were constructed from the clone sequences for PCR analysis of the four rat strains to determine which markers are informative in our parental strains. To measure the length of the SSR, genomic DNA was used as a template for PCR with unique primers. A radiolabeled deoxynucleotide was incorporated during the reaction so that the product can be visualized by autoradiography or phosphorimaging (Molecular Dynamics) after gel electrophoresis. Informative markers (those demonstrating strain polymorphisms) were then be used to score the backcross animals

for linkage to the MCS phenotype.

Linkage analysis of SSR markers

Genomic DNA samples were prepared from either rat tails or spleens of the backcross and parental animals using the standard procedure (Ausubel *et al.*, 1987). SSR markers were obtained from Research Genetics (Huntsville, Alabama). PCR reactions were performed with [α-^{32}P]dATP (3000 Ci/mmole) and electrophoresed on 5% polyacrylamide sequencing gels. For each reaction 50 ng of genomic DNA was amplified in a 5 μlreaction containing 10 mM Tris-CL, pH 8.3 , 1.5 mM $MgCL_2$, 50 mM KCL, 0.01% w/V gelatin, 120 nM of each primer, 200 μM [α-^{32}P]dATP, and AmpliTaq polymerase (0.5-1 U/100 microliters, Perkin Elmer Cetus). The reactions were set up in 96-well plates using a Biomek workstation (Beckman Instruments), overlayed with mineral oil (Sigma) and run on an MJ Research Programable Thermal Cycler PTC-100 using the following cycling conditions: denaturation at 94 C for 3 min followed by 25 cycles of 95 C, 1 min; 55 C, 1 min; and 72 C, 30 sec. A final 72 C extension step was carried out for 5 min. Wet gels were transferred to Whatman 3M paper, wrapped in Saranwrap, exposed to either film or a phophoimager screen (Molecular Dynamics) followed by analysis by eye or the Phosphoimager (Molecular Dynamics) respectively. For qualitative analysis, phenotypes of backcross animals were defined as resistant or sensitive based on the number of tumors formed. This data, together with the microsatellite or SSR data, was subjected to linkage analysis using the MAPMAKER computer program (Lander et al., 1987). Quantitative analysis was also performed with

the program MAPMAKER-QTL (Lander and Botstein, 1989).

Genetically Driven Representational Difference Analysis

Genomic DNA for all backcross animals and parental strains was isolated from frozen spleens using the standard purifications methods (Ausebel *et al.*, 1987). Restriction endonuclease digestions for all three libraries, *Bam* HI, *Bgl* II, and *Hind* III, were carried out using Promega's (Madison, Wisconsin) enzymes and buffer system at 37 C for 16 hours. The resulting fragments were purified by phenol extraction and precipitation in 0.3 M sodium acetate and 2.5 volumes 100% ethyl alcohol. PCR adaptors were ligated to the DNA using T4 DNA ligase (Gibco BRL, Gaithersburg, PA) at 16 C overnight. PCR amplification was carried out using the suggested protocol (Lisitsyn *et al.*, 1994). Twenty individual libraries were constructed and then mixed for creation of the driver library to avoid underrepresentation of any one of the individual backcross animals. The remainder of the procedure was conducted according to the suggested protocol with the following exceptions. All three restriction libraries were hybridized four times to excess driver DNA with the final PCR amplification step being carried out for 38 instead of 30 cycles. The resulting clones were digested with the original restriction enzyme and ligated into the pUC 19 vector (Gibco-BRL, Gaithersburg, PA). Individual clones were obtained following transformation into competent DH5α bacterial cells (Gibco BRL, Gaithersburg, PA) and plating onto LB ampicillin plates (100 μg/ml). Each clone was purified from the bacterial cells using

PCR and the M13 forward and reverse primers under the same conditions as listed above for generation of the driver and tester libraries. Following analysis on 1% agarose gels the clones were radioactively labeled with [α ^{32}P] dCTP using the Megaprime DNA labeling system (Amersham Life Science Inc., Arlington Heights, ILL) according to the manufacturer's directions. Each probe was then analyzed by conventional Southern blot technology for hybridization to restriction-digested COP and WF DNA.

RESULTS AND DISCUSSION

Objective 1. Chromosome-specific library generation and screening.

In the generation of the chromosome 2-specific libraries, problems were encountered with background contamination, and such problems are currently being addressed. Additionally, the 2q1 library that was generated contained very few unique clones, i.e. most were identical. This common clone was sequenced but was not informative between the four rat strains. In spite of these problems, we were able to obtain two additional markers on chromosome 2 from the whole chromosome 2 library. These markers are designated UW5 and UW9, and are included in the updated map shown in appendix A, but neither are linked to the MCS phenotype. As in objective 2 (below), additional SSR markers have been obtained from Research Genetics, and from Dr. Howard Jacob (Mass. General Hospital). Of these, 8 markers have been added to the map shown in our initial proposal. Thus, along with the 2 markers from

our libraries, we have added 10 markers to the rat map. Notably, marker R696 maps 0.6 cM proximal to M13 and is linked to *Mcs-1* with a quantitative LOD of 3.2 (compared with 3.8 for M13). This serves as confirmation of the linkage of M13 to *Mcs-1*. However, more markers are still needed in this region to accurately locate *Mcs-1*. We are continuing to generate libraries from both sorted and microdissected chromosome 2, and we hope to obtain useful markers in the near future.

Objective 2. Testing of SSR markers over entire genome.

Additional microsatellite markers have been obtained from Research Genetics, as well as several unpublished ones from the lab of Dr. Howard Jacob. Those that are informative have been added to our overall map of the genome and have been tested for linkage to the MCS phenotype. The updated genome map shown in the appendix contains 30 new markers compared with the map at the time the proposal was submitted.

Upon testing these markers for linkage to the MCS phenotype, several markers on chromosome 7 appear somewhat promising. Using quantitative analysis, marker R5141 yielded a LOD score of 2.2, and R3543 yielded a LOD of 2.6. Though these LOD scores are less than the preferred cutoff of 3.0, they are worth pursuing. We will continue to try to find additional informative markers in this region to determine whether it may represent an additional quantitative trait locus, i.e. *Mcs-2*. If necessary, we will generate sorted and/or microdissected chromosome 7 libraries to obtain such markers

as is being done for chromosome 2.

This work is ongoing as we still have roughly 63 informative markers to screen, plus an additional 87 uncharacterized markers which, if informative in our backcrosses, can be screened for linkage to the MCS phenotype. Should the need arise, we also have access to some 600-700 unmapped markers which can be tested for informativeness and then both mapped and screened for linkage to the phenotype.

Genetically Directed Representational Difference Analysis

GDRDA was performed using COP DNA for construction of the tester library and a combination of 20 DNAs from backcross animals containing 4 or more mammary tumors for driver library construction. We have completed 4 rounds of hybridization/amplification for 3 separate restriction endonuclease libraries: *Bam* HI, *Bgl*II, and *Hind*III, and are currently in the process of screening clones from each library for RFLP's between COP and WF parental DNA. To date 50 *Bgl*II clones, 36 *Bam*HI clones, and 15 *Hind*III clones have been screened by Southern blot using COP and WF DNA digested with the appropriate restriction endonuclease. Of the 101 clones tested thus far only one has demonstrated a polymorphism between the two strains (see Appendix B). This clone, designated *Bam*20 shows a high molecular weight polymorphism. The polymorphism pattern expected from this procedure is a small fragment in one parental strain and a large fragment in the other. *Bam*20 binds to several fragments in both COP and WF DNA, but does not show the expected pattern.

For this reason, we do not believe that this marker will prove to be linked to the MCS phenotype, however, we are currently working to map this clone and test it for linkage. The GDRDA technique has thus far not proved overly successful for our purposes. Therefore we intend to continue screening clones until we have screened 50 from each of the three libraries. If at that time no useful markers have been obtained, we will abandon this procedure and concentrate on objectives 1 and 2.

CONCLUSIONS

There are few conclusions that can be made at this time other than we need to continue pursuing the experimental methods detailed above to further map the rat genome and identify markers which are linked to the mammary carcinoma resistance phenotype in rats. The fact that we are working in the rat means that progress will be slower since large number of markers are not yet available for use as in the mouse and human.

Thus far however we have located a new site on rat chromosome 7 which may be linked to the resistance phenotype and therefore may represent a second allele, *Mcs-2*. We have worked out the details for use of the GDRDA procedure and as yet cannot conclude if this procedure will provide us with additional markers. Further testing which should be completed by August of 1995, is required to determine the usefulness of this procedure for our purposes. And finally, we continue to screen additional SSR markers for linkage to the MCS phenotype and are continuing our efforts utilizing chromosome-specific and microdissected libraries to isolate new SSR markers in the area of interest

on rat chromosome 2.

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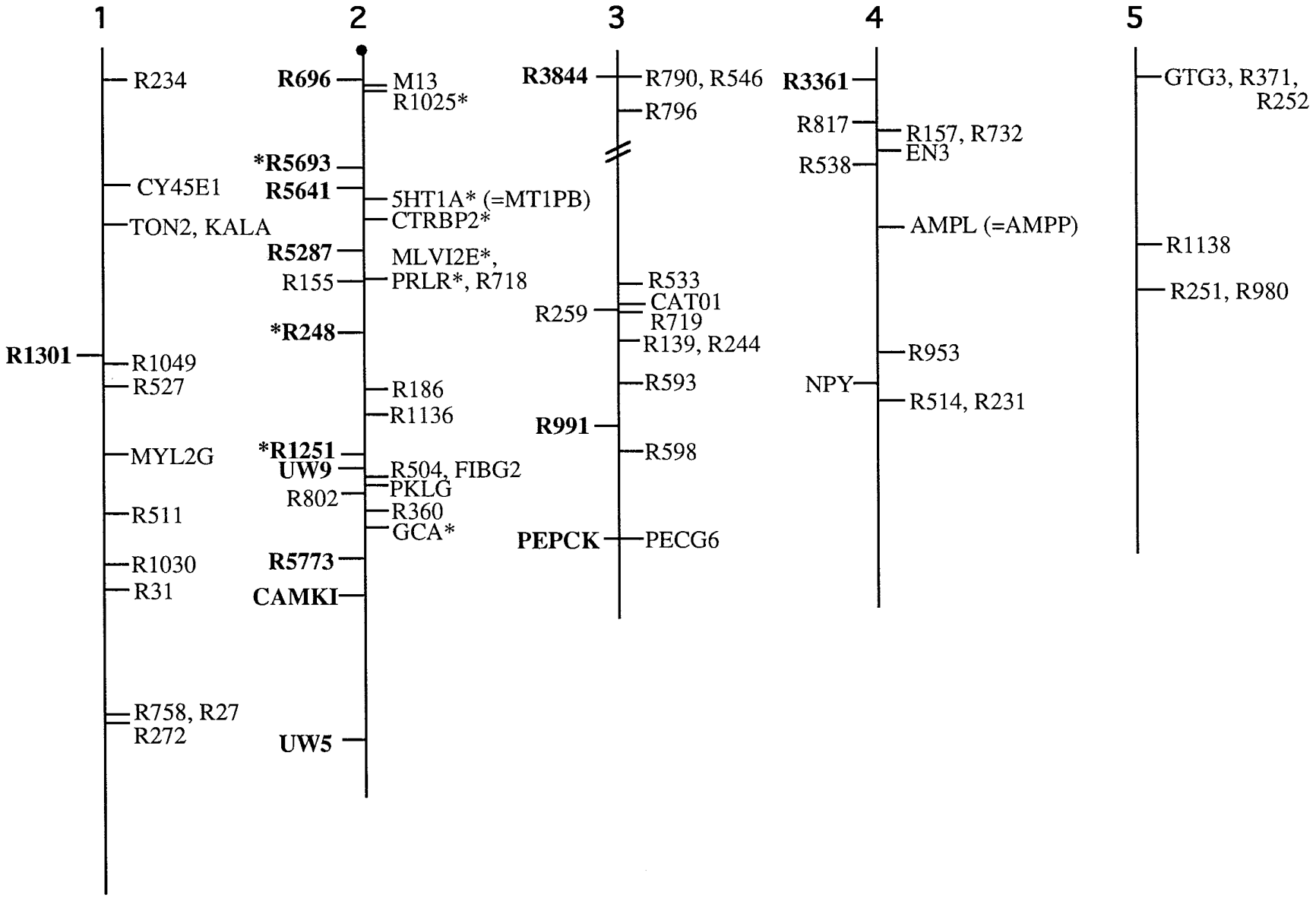
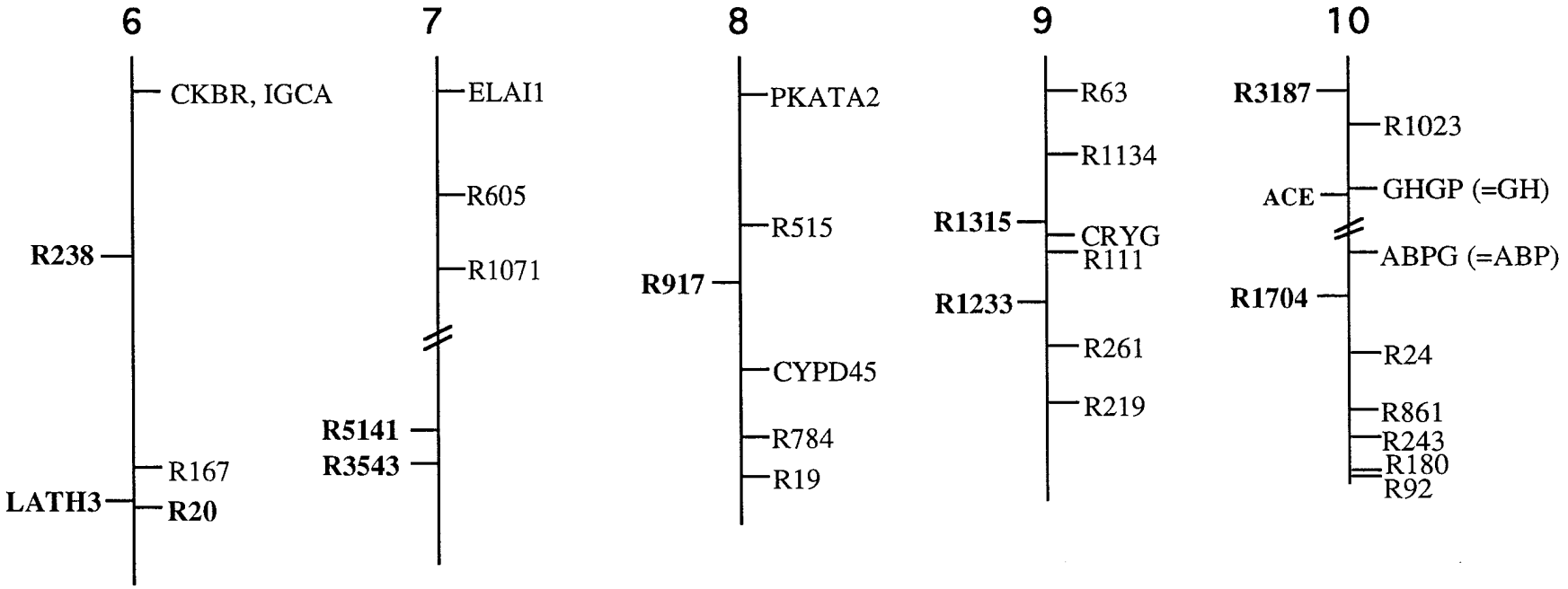
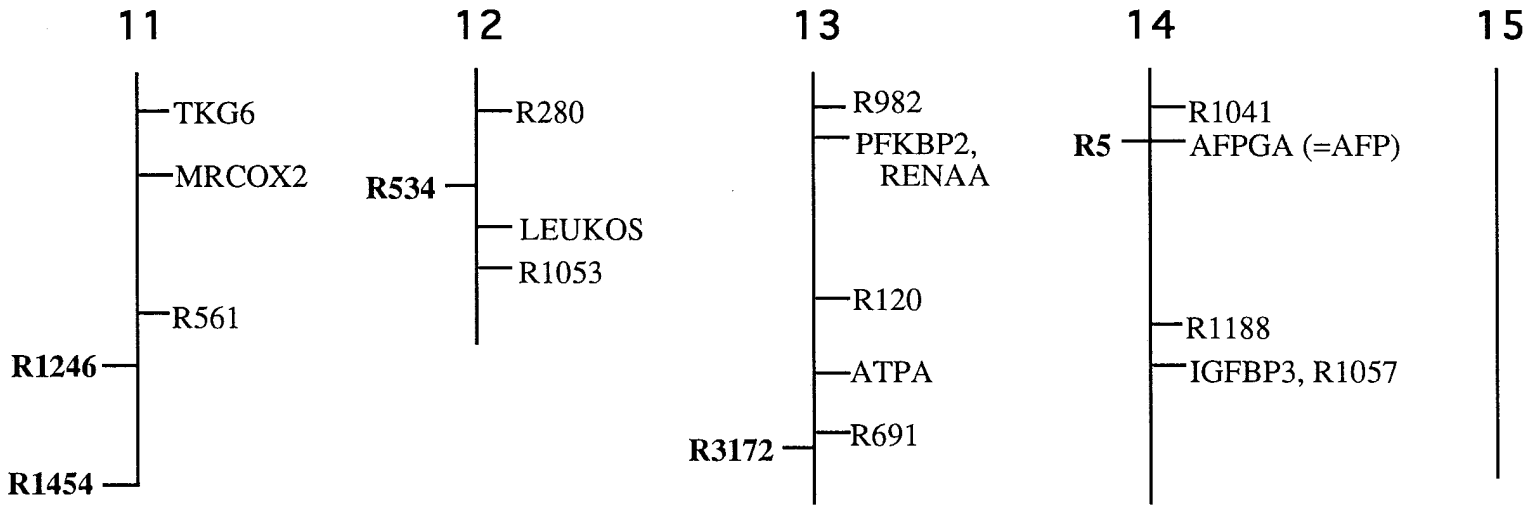
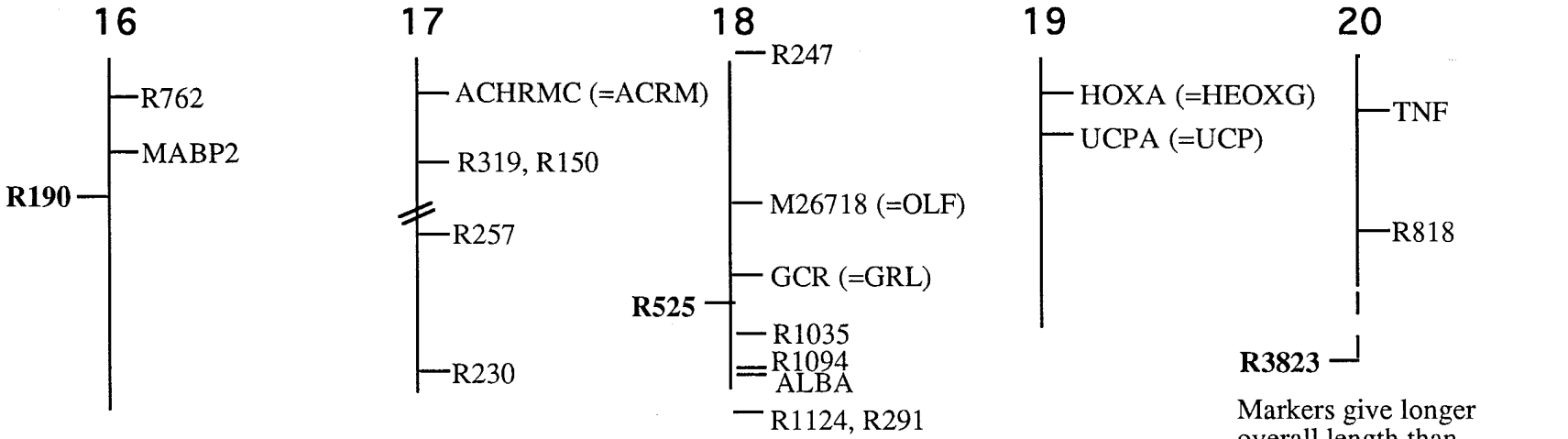
Appendix A -- Genetic map of the rat as determined in the (Cop x WF) x WF backcross




6
CKBR, IGCA
R238
R167
LATH3
R20
7
ELAI1
R605
R1071
R5141
R3543
8
PKATA2
R515
R917
CYPD45
R784
R19
9
R63
R1134
R1315
CRYG
R111
R1233
R261
R219
10
R3187
R1023
ACE
GHGP (=GH)
ABPG (=ABP)
R1704
R24
R861
R243
R180
R92



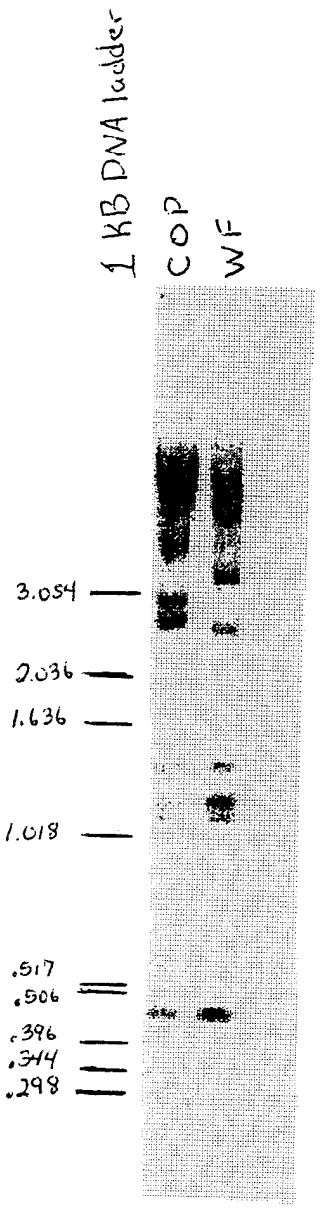
1) The orientation of chromosomes 1 and 2 is known, and are drawn with the short arms at the top. Orientation of the others is not known.
2) The end markers on each chromosome were randomly placed at the same distance from the top of each chromosome line, except for chromosomes 18 and 20 in which the markers indicated a larger map than that predicted by physical measurements.
3) The length of each chromosome is to scale based on physical cytogenetic measurements. If 1cM is not equal to 1 Mbp, then the lines drawn may need to be adjusted.
4) Markers in bold type have been added since the initial grant proposal. Asterisks indicate markers that were mapped in the (F344x WKy) x F344 cross.
5) Double slashes across the chromosome indicate that the distance between markers is not known since they were not linked, but the markers are known to reside on that chromosome based on data from other investigators.



Markers give longer overall length than that calculated by cytological measurements.

Markers give longer overall length than that calculated by cytological measurements.

Appendix B



Genomic Southern blot of COP and WF DNA digested with *Bam*HI and probed with the *Bam*20 clone.